Microfibrils: a cornerstone of extracellular matrix and a key to understand Marfan syndrome

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Microfibrils: a cornerstone of extracellular matrix and a key to understand Marfan syndrome

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Key to abbreviations

BMP	= Bone Morphogenetic Protein	MAGP	= Microfibril Associated GlycoProtein
FBN	= Fibrillin	MFAP	= Microfibril Associated Protein
FBLN	= Fibulin	MFS	= Marfan Syndrome
LAP	= Latency Associated Peptide	SLC	= Small Latent Complex
LLC	= Large Latent Complex	TGF	= Transforming Growth Factor
LTBP	= Latent TGF-β Binding Protein	TGFBR	= Transforming Growth Factor-β Receptor

SUMMARY

The extracellular matrix is made of collagen, reticular, elastic and oxytalan fibers, amorphous ground substance and adhesive proteins playing a structural role, such as fibronectin; the basement membrane is a specialized matrix compartment which adheres to non-connective tissues and is continuous with the remaining matrix thanks to reticular fibers, anchoring fibrils, collagen VI filaments and oxytalan fibers.

Microfibrils are constituents of elastic and oxytalan fibers that confer mechanical stability and limited elasticity to tissues, contribute to growth factor regulation, and play a role in tissue development and homeostasis. The microfibril core is made of the glycoprotein fibrillin, of which three types are known. Other concurring molecules are microfibril associated proteins (MFAPs) and microfibril associated glycoproteins (MAGPs); they, and other peripheral molecules, contribute to link microfibrils to elastin, to other extracellular matrix components and to cells.

Fibrillinopathies are genetic disorders due to mutations in fibrillin genes (*FBN*). The most frequent is Marfan syndrome, caused by mutations in *FBN-1* and involving primarily the cardiovascular, skeletal, ocular and central nervous systems. Several mutations have been identified, which lead to alteration or reduction in the secretion or assembly of fibrillin molecules and to increased microfibril proteolysis. Marfan related disorders are associated with alterations of TGF-β signaling that interfere with extracellular matrix formation. Understanding the pathogenesis of Marfan and related syndromes requires advances in the physiology of the extracellular matrix and in turn can cast light on the roles of microfibrils and of extracellular matrix in general in organ formation and function.

INTRODUCTION

Marfan syndrome (MFS) is a hereditary disorder of connective tissue with autosomal dominant transmission, whose incidence is estimated at 1:5000-1:10000 people. The clinical manifestations involve primarily the cardiovascular, skeletal, ocular and central nervous systems; respiratory and tegumental systems show a minor involvement (Pyeritz, 2002). This pleiotropic disease shows great variability in intra- and inter-family clinical presentation and belongs to a group of hereditary microfibrillopathies called fibrillinopathies type I or Marfanoid diseases. In most patients (90%), mutations in the fibrillin-1 gene (*FBN1*) are the basis of the molecular defect of MFS type 1. A direct consequence of the genetic variation of MFS is an altered or reduced secretion of fibrillin molecules or a derangement in the organization of these molecules into microfibrils, eventually interfering with the formation of elastic fibers of connective tissue. Some cases of MFS-related disorders are associated with mutations of two other genes, *TGFBR1* and *TGFBR2*, coding for receptors of TGF-β and whose mutations seem (paradoxically) to lead to an increase in the cytokine signal; the alteration of TGF-β signal may contribute to the pathogenesis of the syndrome by interfering with the proper formation of the extracellular matrix (Mizuguchi and Mtsumoto, 2007). The relationships between fibrillin and TGF-β are complicated because fibrillin is able to bind TGF-β through complex interactions, thus depressing the cytokine signaling. A vicious circle may onset, inasmuch the lack of fibrillin leads to an increase in TGF-β signal and this in turn interferes adversely with the formation of microfibrils; these reciprocal interactions may explain the similarities in the anatomo-clinical picture between patients with different genetic defects.

Electron microscopical observations suggest that the molecular alterations of MFS bear wide consequences to the extracellular matrix organization, beyond microfibrils, therefore this disease represents both a challenge and a model to understand the structure and function of extracellular matrix and in particular of microfibrils, which are the object of this review.

THE EXTRACELLULAR MATRIX

The extracellular matrix is a composite substance basically made of a polysaccharide gel, the amorphous ground substance which consists of water, proteoglycans and glycoproteins, and embeds predominantly proteinaceous fibers; cells are hosted in the matrix and regulate its composition and properties. The components of the extracellular matrix are produced by connective tissue cells (fibroblasts, condroblasts, osteoblasts), by smooth muscle cells and in part also by cells of other tissues that secrete the components of basal laminae, i.e. collagen type IV, laminin, other non-collagenous glycoproteins and glycosaminoglycans (Aumailley and Gayraud, 1998). The extracellular matrix determines the histological architecture

of organs, provides mechanical scaffolding for cell adhesion and migration, and delivers information to regulate cell proliferation, differentiation, function and survival (Krieg and LeRoy, 1998). The composition of the matrix is extremely variable in relation to the function of different tissues: it ensures such diverse characters as the resilience of cartilage and bone, the tensile strength of tendons, the elasticity of blood vessels and the transparency of the cornea.

Collagen and reticular fibers

Collagen and reticular fibers are made of fibrils (or "microfibrils") glued by intrafiber ground substance; fibrils in turn are formed by regularly arranged collagen molecules. Collagen fibers are highly resistant to tension, while reticular fibers are less resistant to distension and form loose meshes that link the basal lamina to the remaining extracellular matrix or build a tridimensional scaffold where hematopoietic and lymphoid cells are hosted. The mechanical and biological differences between these two fiber types depend largely on the intrafiber ground substance, besides collagen molecules (Ottani et al., 2002; Ushiki, 2002).

Elastic and oxytalan fibers

Elastic fibers are distributed in many connective tissues associated with collagen fibers. Elastic fibers are highly extensible and, as the name implies, elastic, i.e. they recover the original size and shape at the release of tension. These fibers are particularly numerous in elastic tissue, which is found in the tunica media of blood vessels, most abundant in arteries, and in intervertebral yellow ligaments; elastic fibers are especially abundant also in the lung stroma and the skin. The diameter of individual elastic fibers ranges from a few tenth of microns to a few microns. They may extend and connect with each other, coalesce to form fenestrated laminae, in particular in blood vessel wall, or arrange in parallel bundles to form elastic ligaments. Elastic structures (elastic fibers, elastic laminae and elastic ligaments) have a microfibrillar component at electron microscopy, consisting mainly of fibrillin, and an amorphous component formed by elastin, an elastic aggregate the monomer of which is tropoelastin. Three types of fibrillin molecules have been identified, fibrillin-1, fibrillin-2 and fibrillin-3. The microfibrils are mainly disposed around an elastin core and in part inside this core. Small bundles of microfibrils can be found without the elastic matrix, forming so-called oxytalan fibers. Oxytalan fibers connect elastic fibers to other structures (such as the lamina densa of basement membrane, or muscle cells of the arterial wall) and can be found without any relation to an elastic matrix in certain locations, such as in periodontal tissues and in the zonular fibers of the eye lens. Oxytalan fibers are not elastic, but rather resist to tension (Kielty, 2006). In addition, two other molecules have been identified in the skin elastic fibers by immunohistochemistry: vitronectin and amyloid P protein (Dahlback et al., 1990); other sites have not yet been investigated in this respect.

Amorphous ground substance

The ground substance, interposed between fibers and located in part also inside collagen fibers to glue fibrils together, is a gel made of hyaluronic acid, proteoglycans and glycoproteins as well as water, electrolytes and other solutes. Proteoglycans are a highly heterogeneous group of molecules formed by a protein filament to which many polysaccharide side-chains are inserted, made of glycosaminoglycans. The latter molecules consist of disaccharide units repeated many times in a linear sequence; each dimer is formed by a monosaccharide or an uronic acid, and by an amino sugar; glycosaminoglycans may be more or less extensively sulfated. Hyaluronate is the only glycosaminoglycan which is not sulfated and may not even be associated with proteins; when it is present with proteoglycans (which is the most frequent condition), it forms a central axis to which the protein chains of proteoglycans are connected, which in turn give insertion to glycosaminoglycans. The high content in alcoholic and acidic groups makes proteoglycans highly soluble and suitable to provide for plasticity and flexibility and, at the same time, considerable resistance to compression forces. The direct interactions between cells and the components of the extracellular matrix can be strengthened by interstitial glycoproteins, namely laminin and fibronectin, which are recognized by specific integrin receptors at the cell surface (Heino et Käpylä, 2009).

Fibronectin filaments

A major glycoprotein in the ground substance is fibronectin, which is present in both the pericellular and interstitial matrix. It is a homodimer, the two polypeptide chains are linked by disulfate bridges and contain domains responsible for interactions with other molecules of fibronectin, collagen, heparin and cells. Fibronectin exists in two forms: intercellular fibronectin present in tissues where it is assembled into a fibrous network, and soluble plasma fibronectin, which polymerizes upon blood vessel injury (Sabatier et al., 2009). Once secreted, fibronectin may also polymerize into filaments; the assembly of this, as well as other intercellular filaments and fibrils, is controlled by cells, which determine in this way the mechanical properties of the surrounding extracellular matrix (Mao et Schwarzbauer, 2005).

The so called fibronectin type III domain, which is characterized by the sequence arginine-glycine-aspartic acid ("RGD"), is responsible for interaction with cellular integrin receptors. Fibronectin molecules induce receptor aggregation with subsequent recruitment of cytoskeletal (such as talin) and signaling molecules (such as Src and Fak). These molecular interactions are followed by a reorganization of the actin cytoskeleton and the further aggregation of other proteins to form large aggregates on the cytoplasmic side of the cell membrane, named focal adhesions. The activity of actin cytoskeleton in turn induces a change in the conformation of fibronectin promoting its aggregation into fibrils (Mao et Schwarzbauer, 2005; Sabatier et al., 2009).

Fibronectin and fibrillin-1 are colocalized in the extracellular matrix produced by dermal fibroblasts, at least in an early stage of matrix formation and fibronectin must be expressed by fibroblasts as a prerequisite for the organization of fibrillin-1 into a fibrillar matrix, hence interaction between fibrillin-1 and fibronectin appears crucial for the assembly of microfibrils. This assumption is supported by the fact that a network of exogenously added fibronectin promotes the formation of microfibrils, through interactions which occur either on, or close to, the cell surface (Sabatier et al., 2009).

Basement membrane

The basement membrane is a thin layer of extracellular matrix, between 70 and 300 nm thick, that separates the epithelium from the connective tissue and is found, with similar features, any time the extracellular matrix is in contact with a non-connective tissue; it provides physical support to cells and regulates the microenvironment acting as a sieve. It also gives adjacent cells clues for their survival, localization and function. The basement membrane is composed of three layers. The lamina lucida, which is adjacent to the unit plasma membrane, is transparent to electrons and corresponds to the glycocalix of the cell, containing the binding sites of the glycoproteins for cell-matrix adhesion. The lamina densa is the central layer, is opaque to electrons and contains collagen type IV, non-collagen glycoproteins – the principal one of which is laminin – and proteoglycans; these molecules are secreted by the epithelial cell but may also be secreted by the connective tissue cell. These first two layers together form the basal lamina. The lamina reticularis, or reticular lamina (also designated as sublamina densa), connects the lamina densa to the remaining extracellular matrix and is rich in fibrous components including reticular fibrils, anchoring fibrils (consisting of collagen VII) and fibrillin microfibrils; the molecules for this lamina are secreted by connective tissue cells. Among others, a basement membrane of variable thickness is interposed between the epidermis and dermis, where it provides a transition between a semi-rigid structure consisting only of cells, namely the epidermis, and a deformable and elastic structure which consists mainly of fibers and jelly-like components, the dermis (Breitkreutz et al., 2009).

Connecting systems

Fibers of the extracellular matrix and basal laminae are connected with each other by various structural systems. Major systems include anchoring fibrils, collagen VI filaments and oxytalanic fibers.

Anchoring fibrils are composed of thin bundles of collagen VII, whose ends are embedded in the basal lamina or in anchoring plaques; the latter are small densities in the lamina reticularis similar to the lamina densa and consisting predominantly of collagen IV. The network of anchoring fibrils embraces bundles of collagen fibrils,

thereby establishing an effective integration between the basal lamina and interstitial stroma (Villone et al., 2008).

Collagen VI forms beaded filaments throughout the extracellular matrix which are especially abundant in the lamina reticularis. They form a mesh which binds both collagen IV in the basal lamina and interstitial collagen fibers (van der Rest and Garrone, 1991).

The integration between the basal lamina and elastic fiber is provided by oxytalan fibers, which can insert into the basal lamina and towards the interstice merge with elastic fibers with the intermediate of so called elaunin fibers, which are made of microfibrils and a small amount of elastin (Montes, 1996).

The cells establish an intimate contact with the extracellular matrix through specific receptors. The most important group of these molecules are integrins, which are heterodimeric transmembrane glycoproteins made of two different transmembrane proteins identified an α and a β subunit; their extracellular portion binds the extracellular matrix, while their cytoplasmic portion interacts with multiple cytoskeletal components (Barczyk et al., 2009).

MICROFIBRILS

As already mentioned, in addition to collagen fibers, elastic fibers, proteoglycans and non fibrillar glycoproteins, microfibrils are important constituents of the extracellular matrix (fig. 1); their functions are dependent on their composition, the stage of development in which they are synthesized and the tissue where they are expressed. When microfibrils are associated to elastin, they act as both a scaffold for the organization of elastic fibers and a link between the latter and cells. Usually microfibrils appear as a "cloak" around elastin (Rantamaki et al., 1997). In tissues where microfibrils are found without any relationship to elastin matrix, they form an anchor between other tissue components, as in the eye between the lens capsule and the ciliary body (Cleary and Gibson, 1996) or the skin between the basal lamina and the deeply located elastic fibers (Kielty and Shuttleworth, 1997; Tiedemann et al., 2005). In mature elastic fibers, microfibrils are present at the fiber periphery and may also be embedded within the crosslinked elastin core (Kielty et al., 2002).

Fibrillins

The major component of microfibrils is fibrillin, a glycoprotein. The family of fibrillins includes three members, fibrillin-1 and fibrillin-2, encoded respectively by *FBN1* gene located on chromosome 15q21 and *FBN2* gene on chromosome 5q23 (Pereira et al., 1993), and fibrillin-3, which is encoded by *FBN3* gene located on chromosome 19p13; fibrillin-3 has been until now studied at the nucleic acid level: it shows a high homology to the other family members and its expression is highest in brain tissue (Nagase et al., 2001; Corson et al., 2004; Uyeda et al. 2004). Fibril-

lin-3 has not yet been implicated in extracellular matrix disorders nor in other diseases (Prodoehl et al., 2009). Fibrillin-1 (fig. 1) weights 330 kDa and is a component of the 10-12 nm thick microfibrils localized in elastic tissue (aorta, skin, periosteum) and non-elastic tissue (ciliary zonula) (Sakai et al., 1986). Fibrillin-2 weights 350 kDa and is preferentially localized in the extracellular matrix rich in elastic fibers. Fibrillin monomers interact with a head-tail orientation to form a multimeric complex, which in combination with other matrix proteins forms microfibrils (Dietz and Pyeritz, 1995). Ultrastructural studies of fibrillin monomers show "beads-on-string" structures, with a diameter of 10-12 nm and a regular periodicity between the beads of 50-55 nm (Dietz and Pyeritz, 1995; Kielty and Shuttleworth, 1997). These microfibrils, in many cases, form a continuous link between the cells and the extracellular matrix which suggests that they facilitate the assembly of elastic fibers and also work by anchoring cells to extracellular components, since fibrillin is a valuable adhesion protein (Sakamoto, 1996). Fibrillin is primary resilient to tension but is endowed with some elasticity itself, although by no means comparable with elastin (Kielty et al., 2002). The gene expression of the two fibrillins begins during embryogenesis, *FBN2* is expressed first, except in the cardiovascular system, and seems to be very important in elastogenesis (Dietz and Pyeritz, 1995). *FBN1* is expressed also in areas with microfibrils that are not associated with elastic fibers. In elastic arteries (i.e. aorta), fibrillin-1 is found in all three layers of the wall, while fibrillin-2 is concentrated in the media (Dietz and Pyeritz, 1995). The findings in the aorta suggest that fibrillin-2 is not found where there are no elastic fibers, but cannot lead to conclude that it is predominantly expressed in microfibrils associated to elastic fibers.

Fibrillins are structurally related to the LTBP (latent TGF-β binding protein) gene family and can bind LTBP. Until now, four LTBP genes have been identified. LTBP-1, in particular, undergoes rapid intracellular association with TGF-β, which increases the secretion of TGF-β from cells. This complex is able to store latent TGF-β in the extracellular matrix; the release of this cytokine from the matrix as a result of proteolytic cleavage of LTBP-1 is one possible mechanism for the regulation of TGF-β action. LTBP-1 and TGF-β co-localize with fibronectin, collagen type IV and fibrillin. Like LTBP-1, also the remaining three isoforms can bind latent TGF-β and appear to undergo association with other extracellular matrix molecules (Dallas et al., 1995).

As already anticipated, *FBN1* and *FBN2* are differentially expressed both in terms of developmental stages and tissue distribution. In most cases, developmental expression of the fibrillin genes exhibits a biphasic pattern, with the onset of *FBN2* expression occurring earlier than *FBN1* expression. *FBN2* transcripts seem to accumulate before tissue differentiation and to decrease rapidly or disappear thereafter; *FBN1* transcripts increase at a gradual rate thereafter. As anticipated, fibrillin-2 is found preferentially in elastic tissue, such as the elastic cartilage, the tunica madia layer of the aorta, and along the bronchial tree (Robinson and Godfrey, 2000). The two fibrillins may therefore have differing functional roles; it has

been proposed that fibrillin-2 regulates the early process of elastic fiber assembly, whereas the predominance of fibrillin-1 in stress and load bearing structures, like the aortic adventitia, the ciliary zonules and the skin, suggests that it may be mainly responsible for the stability of microfibrils especially against tensile load (Charbonneau et al., 2003).

The clinical phenotypes resulting from mutations in the two respective genes are different and distinct. Mutations in *FBN1* are associated with MFS and more generally to "fibrillinopathies type I". Mutations in *FBN2* are responsible for congenital contractural arachnodactyly, a disorder characterized by articular and skeletal abnormalities (Beals and Hecht, 1971; Pyeritz, 2000).

Other microfibril proteins

There is more than fibrillin in microfibrils (fig. 1). Other integral components of microfibrils are microfibril-associated proteins (MFAP). Microfibril-associated protein 1 (MFAP-1) maps to the same region of *FBN1*, on chromosome 15q15-q21; MFAP-2 is an earlier designation for MAGP-1 (see below); MFAP-3 gene is located near to the locus of *FBN2*; MFAP-4 is most abundant in the aortic adventitia. This glycoprotein has a fibrinogen-like domain and a RGD cell adhesion motif in its N-terminal region.

Two other glycoproteins found in microfibrils have been named microfibril associated glycoprotein 1 and 2 (MAGP-1 and MAGP-2). The first appears to be covalently bound to microfibril by intermolecular disulfide links; its N-terminal region can bind both tropoelastin and type VI collagen, it is possible that MAGP-1 is responsible for the organization of tropoelastin monomers in the extracellular matrix before their cross linking. MAGP-1 localizes specifically to the beads of isolated microfibrils, and several MAGP-1 molecules may be present in a single bead. MAGP-2 is also disulfide-bonded to fibrillin-containing microfibrils. MAGP-2 presents an RGD cell adhesion motif in its N-terminal domain which can interact with a range of cell types via $\alpha_v\beta_3$ integrin. MAGP-2 has a more restricted tissue and developmental distribution than MAGP-1, which may reflect a different functional role (Brown-Augsburger et al., 1994).

The fibulins form a recently characterized family of extracellular matrix proteins with several unique structural features. Pan et al. (1993) have described differences in the expression pattern between fibulin-1 and fibulin-2, while Zhang et al. (1995) found that this pattern is similar in the developing heart. Fibulin-1 is present in basement membranes and in the amorphous core of elastic fibers, but not in individual microfibrils. It may be suggested that this protein is involved in organizing basement membrane and microfibrils in angiogenesis and capillary formation. Another matrix protein, fibulin-2, codified by *FBLN2* gene located on chromosome 3p24-25, interacts with fibrillin-1 perhaps stabilizing the relationship between basal lamina and microfibrils (Reinhardt et al., 1996). This protein is highly expressed in the vessel walls, and alterations in fibulin-2 gene are often associated with dis-

ease (in particular aneurisms) (Pan et al., 1993, Zhang et al., 1995). Fibulin-2 co-localizes with fibrillin-containing microfibrils in some tissues, like skin (Reinhardt et al., 1996); this protein binds fibronectin and tropoelastin strongly and with high affinity and may also play a connecting role between several proteins of the extracellular matrix such as laminin, nidogen, fibrinogen and fibronectin. As all other fibulins, fibulin-3 is present in blood vessels of different sizes. Fibulin-3 is capable of inhibiting vessel development and angiogenesis; it is expressed in cartilage and bone structures during development and may play a role in the skeletal system (El-Hallous et al., 2007). Fibulin-4 is preferentially found in the microfibrils surrounding the elastin cores, it is necessary for elastic-fiber formation, since its absence abolishes normal elastogenesis and leads to irregular elastin aggregates; it also can interact with fibrillin. Fibulin-5 is present at elastic-fiber-cell interface; it can interact with both fibrillin-1 and tropoelastin and has been proposed to play a role in the deposition of tropoelastin onto microfibrils and in elastogenesis (Yanagisawa et al., 2002; Wagenseil and Mecham, 2007). This protein is expressed by vascular smooth muscle cells and endothelial cells, mediates vascular cell adhesion through integrin receptors, influences smooth muscle cell proliferation and migration, and regulates elastic fibrogenesis. In the aorta, it localizes on the surface of the inner elastic lamina adjacent to endothelial cells and throughout the media (Nakamura et al., 2002; Kielty, 2006).

Laminin is found in basement membranes and plays a crucial role in the supramolecular organization of these structures (Timpl and Brown, 1994); it shows calcium-dependent self assembly, and heterotypic binding of laminin to perlecan, nidogen, fibulin-1, heparin and other matrix components forms a highly crosslinked basement membrane (Durbeej, 2009). Since fibrillin-containing microfibrils insert into basement membranes, laminin may play a role in protein-protein and cell-microfibril interactions, however that role should be indirect since laminin has not yet been shown to interact directly with fibrillin.

Perlecan is an intrinsic constituent of basement membranes but is also present in other locations in the extracellular matrix; it has three to five heparan sulfate chains attached to the protein core (Melrose et al., 2008), although one or more of these chains may be substituted for by other glycosaminoglycans (French et al., 2002; Govindraj et al., 2002; Knox et al., 2005). The perlecan core protein is known to interact with a number of extracellular, cellular and soluble proteins like nidogen, fibulin-2, fibronectin, laminin, cell surface molecules and growth factors (Costell et al., 1999; Melrose et al., 2008). Perlecan can interact with fibrillin-containing microfibrils in the vicinity of basement membranes. Tiedemann et al. (2005) have demonstrated that there is a direct and high affinity interaction between fibrillin-1 and perlecan and that this interaction may be involved in anchoring microfibrils to basement membranes; furthermore perlecan may function to connect fibrillin-1 to fibronectin fibers in the early stages of microfibril biogenesis.

Other proteins co-localize or are associated with fibrillin-containing microfibrils. Emilins (*e*lastin *m*icrofibril *i*nterface *l*ocated prote*in*s) are extracellular matrix

glycoproteins, they are found in blood vessels and in the connective tissue of different organs, always in association with elastic fibers containing elastin (Colombatti et al., 2000; Verdone et al., 2009). There are three homologous proteins that constitute the protein family of emilins. Recently it was found that the secreted protein emilin-1 is a negative regulator of TGF-β signaling (Zacchigna et al., 2006). It appears early in embryogenesis and may be involved in elastogenesis (Wagenseil and Mecham, 2007).

Microfibrils that contain fibrillin have been found associated with a chondroitin sulphate proteoglycan, the nature and composition of which is not known. Since the expression of the small proteoglycan decorin may be altered in neonatal MFS, decorin is a possible candidate to this role. Decorin seems to be present in tissues only as a matrix molecule; it binds collagen fibrils at the "d" or "e" bands and "decorates" the fibrils, hence its name; it also acts as a bridging molecule between type I and type VI collagen; in mice, the lack of decorin leads to abnormal fusion of collagen bundles and to increased skin fragility (Seidler and Dreier, 2008).

Molecular interactions at the microfibril surface

In vitro assays have revealed that fibrillin-1 can interact with many different matrix molecules (fig. 1). The N-terminal region is particularly sticky, interacting with high affinity both homotypically and with MAGP-1 and MAGP-2; also, this region of the molecule can react with other structural molecules of the extracellular matrix, precisely with fibulin-2 and 5, lecticans (a family of proteoglycans), heparin, and with regulatory molecules stored in the extracellular matrix, precisely LTBP-1, chondroitin sulphate proteoglycans and BMP-7. The central region of fibrillin-1 contains binding sites for tropoelastin, heparin, versican (a large aggregating proteoglycan that co-localizes with the elastic network of the dermis in human skin) and perlecan. The C-terminal region of fibrillin contains two binding sites for heparin.

Tropoelastin can be covalently linked to fibrillin-1 by transglutaminase. Heparan-sulphate, MAGP-1, decorin, fibulin-5 and biglycan (an extracellular matrix proteoglycan consisting of an axle and two side glycosaminoglycan chains of chondroitin or dermatan-sulphate) also bind tropoelastin, but their binding sites and type of links have not yet been identified.

Molecular interactions are relevant to elastogenesis. During this process, microfibril formation begins before elastin matrix deposition. Elastin, in the form of soluble tropoelastin molecules or small elastin aggregates, becomes associated with pre-formed microfibrils. Fibulin-5 can interact with both fibrillin-1 and tropoelastin and may play a role in the deposition of tropoelastin onto microfibrils. It is not known how other molecules present at the elastin-microfibril interface, such as fibulin-2 and 4 and emilin-1, might influence this process. Immunofluorescence in tissues demonstrated colocalization of fibrillin and fibulin-2 in skin, perichondrium, elastic intima of blood vessels, and kidney glomerulus, while fibulin-2 is not present in ocular ciliary zonular fibrils, tendon, and the connective tissue around kidney tu-

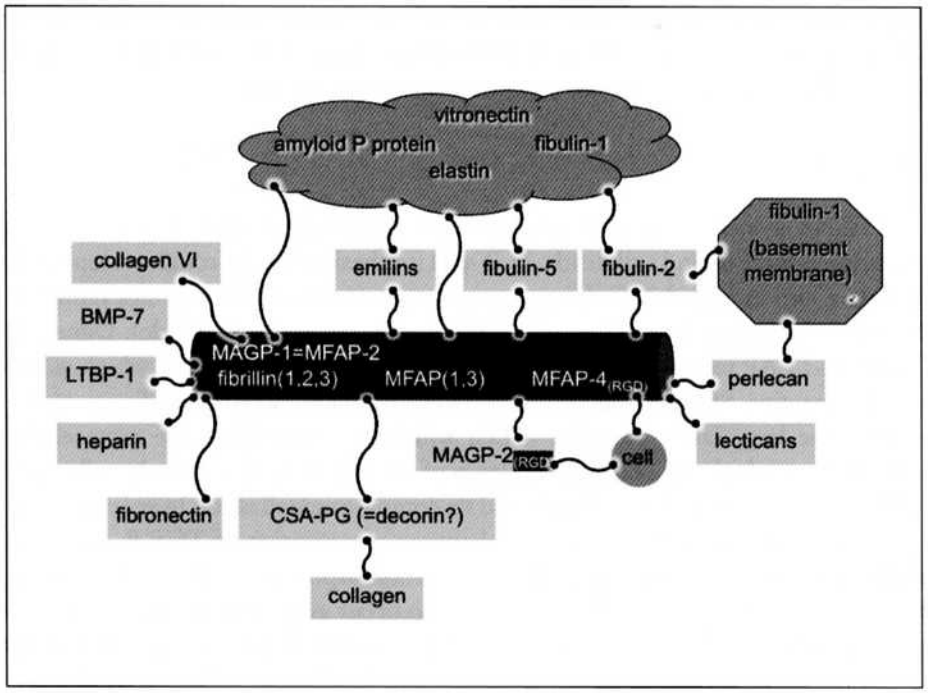

Fig. 1 — Extracellular matrix: schema of the interactions between microfibrils, elastic fibers, basement membrane and cells. Vitronectin and amyloid P protein have until now been identified only in the elastic fibers of the skin.

bules and lung alveoli, which all contain fibrillin. Immunogold labeling of fibulin-2 on microfibrils in skin was found preferentially at the interface between microfibrils and the amorphous elastin core, suggesting that in vivo the interaction between fibrillin-1 and fibulin-2 is regulated by cellular expression and secretion as well as by protein-protein interactions (Reinhardt et al., 1996) and that fibulin-2 may also play a role in the deposition of elastin onto microfibrils.

Deposited elastin is stabilized by lysyl-derived crosslinks formed upon oxidation of residues by lysyl oxidase (LOX) and/or lysyl oxidase-like (LOXL) enzymes, which may also interact with microfibrils.

Ultrastructural analysis has shown that the contribution of fibrillin-1 is essential for the structural integrity of the connecting filaments between elastic laminae and smooth muscle cells in the arterial wall, and that fibrillin-2 or other elements of connective tissue can not compensate for this absence. It was also suggested that fibrillin has not only a role in relation to elastic fibers but even a primary general role in extracellular matrix organization; this is confirmed by data obtained from mutant mice that have a marfanoid phenotype (Bunton et al., 2001).

Surprisingly, studies on fibrillin-1 deficient mice and data on patients with MFS show that microfibrils can be formed, with apparently normal appearance,

despite shortage or even absence of fibrillin. Bunton et al. (2001) have shown that not only heterozygous patients with MFS but also homozygous mice lacking fibrillin-1 have only a partial, and not complete loss of microfibrils.

TGF-β

TGF-β is a cytokine implicated in many cell functions such as proliferation, migration, synthesis and cell death. TGF-β is synthesized in the rough endoplasmic reticulum and modified in Golgi apparatus; some synthesized molecules remain at the stage of propeptide and form the LAP (latency associated peptide). Dimers of TGF-β and LAP form a complex called SLC (small latent complex), which is biologically inactive. SLC is covalently linked to LTBP (latent TGF-β binding protein), forming the large latent complex (LLC) which is secreted into the extracellular matrix; the gradual and controlled release of TGF-β from this complex allows to regulate the activity of this molecule and its compartmentalization in specific areas. There have been several proposed mechanisms of activation of TGF-β, including interaction with proteolytic enzymes, with thrombospondin -1 and with integrins; the release of active TGF-β from LLC can also occur as a result of interaction with ionizing radiation. The activation of TGF-β in vivo and the control mechanisms of this process are not yet clear (Kaartinen and Warburton, 2003). It seems that LTBP complex physically interacts with FBN1, which sequestrates LLC thus limiting TGF-β activation. It has been proposed that the lack of microfibrils caused by a defect in FBN1 alters the binding and the partitioning of latent TGF-β and leads to an increased availability and activation of this molecule, which would inhibit tissue growth. The domain of fibrillin that binds TGF-β1 (LTBP-4) plays a structural role, since its elimination causes a severe defect in the structure of elastic fibers, but the possible role of TGF-β in this defect is unknown.

It is not easy to reconcile these data, indicating a negative role of TGF-β on the synthesis of extracellular matrix in areas rich in elastic fibers, with the pro-fibrotic role recognized to this cytokine and with the stimulus that, according to some studies, it seems to carry on the production of microfibrils themselves. Kissin et al. (2002) have shown that the addition of TGF-β to cultured human skin fibroblasts stimulates the formation of fibrillin although it apparently does not alter the synthesis or translation of the mRNA for this protein. The authors put this fact in relation to the induction of a myofibroblastic phenotype by TGF-β, without advancing any hypotheses on the mechanism underlying this relationship. Conversely, in the fibroblasts of patients affected by diffuse scleroderma the inhibition of TGF-β receptor type I reduces down to a complete loss the cell adhesive capacity and contractility and, in parallel, the expression of fibrotic markers such as collagen type I and integrin β1 (Chen et al., 2006). Also in TGF-β1 knock-out mice there is marked reduction in collagen deposition, together with signs of generalized inflammation and tissue necrosis (Leask, 2008).

GENETICS OF MARFAN SYNDROME

MFS is an autosomal dominant disorder with an estimated incidence of 1:5000-1:10000 individuals that shows marked inter- and intra- familial variability (Judge and Dietz, 2005). This syndrome shows complete penetrance and more than 25% of the cases are the result of new mutations. Cases of germline and/or somatic mosaicism have been reported. Mutations are scattered througout *FBN1* and there seems to be no obvious correlation between clinical pheotype and localization of the mutation.

The gene *FBN1* contains 65 exons spanning 235 kb of genomic DNA (Biery et al., 1999) and consists primarily of repetitive motifs; most mutations occur within the 47 tandem epidermal growth factor-like domains (fig. 2) and many disrupt one of the six predictably spaced cysteine residues, that interact via disulfide linkage to determine domain folding, or residues that affect specifically the calcium binding EGF-like domains of fibrillin-1 (cbEGF-like). A cbEGF-like domain in fibrillin is about 42 amino acids long and typically contains 6 cysteines. These residues are coupled in pairs through disulfide bridges, favoring the formation of antiparallel β-sheets. The formation of a β-sheet may facilitate the interaction with calcium and that between adjacent cbEGF-like domains and explain how they can operate as a single functional unit. cbEGF-like domains are covalently linked to each other; NMR analysis shows a rigid rod conformation, stabilized by bound calcium and hydrophobic interactions (Corson et al., 1993, Pereira et al., 1993). Calcium mediates

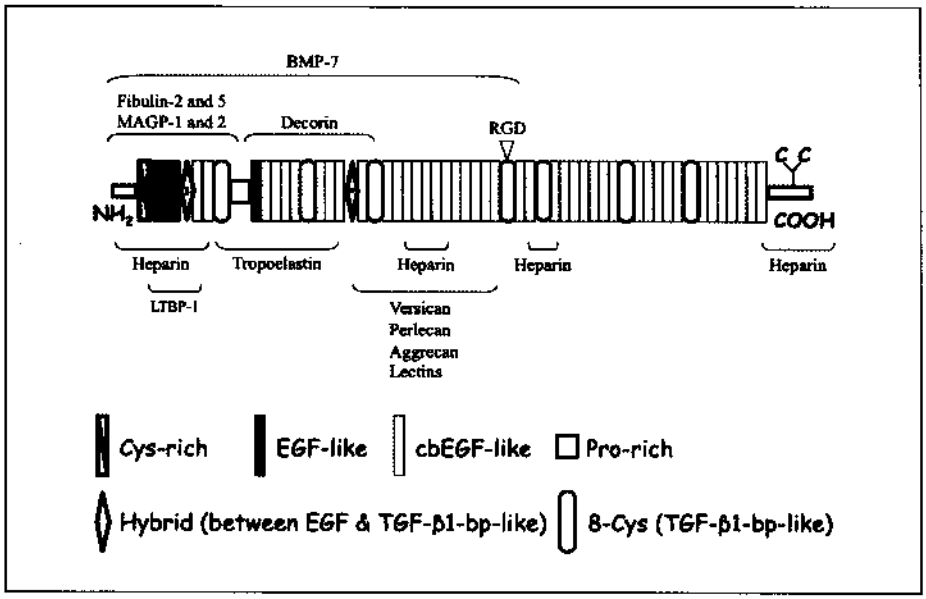

Fig. 2 — Diagram of the domain sequence (redrawn after Pereira et al., 1993) and binding sites of fibrillin-1.

protein-protein interactions, play a role in the maturation of the fibrillin precursor and stabilize fibrillin molecule antiparallel β structure (Downing et al., 1996); they also stabilize microfibrils through cross-links between monomers (Handford et al., 1995) and protect them from proteolytic degradation (Reinhardt et al., 1997). Calcium removal leads to increased flexibility between cbEGF-like domains, to a decrease in the length of the interbead regions and to an increase in the diameter and overall flexibility of microfibrils (Downing et al., 1996; Cardy et al., 1998; Smallridge et al., 2003); perturbations of the cbEGF-like domain structure lead to enhanced proteolytic degradation of fibrillin (Reinhardt et al, 1997). Most of the known mutations in Marfan syndrome affect cbEGF-like motifs and destroy the link with Ca^{2+} in two ways: either changing an aminoacid directly involved in the binding, or replacing a cysteine with another aminoacid with the result of an odd number of cysteine residues interfering with the stabilization of β-sheets.

The relationship between fibrillin and calcium is intriguing in view of the possible calcification of elastic fibers which occurs in mice harbouring a mutation of fibrillin itself (Bunton et al., 2001) and in dystrophic cutaneous calcification. Elastic fibers calcification is a hallmark of pseudoxanthoma elasticum, a condition due to inactivation of the ABCC6 transporter gene through obscure pathogenetic mechanisms, but no obvious alteration of fibrillin deposition has been found in this condition (Godfrey et al., 1995; Chassaing et al., 2005). Calcification of elastic fibers starting from the fiber surface, where microfibrils occur, has been seen in a mouse model of pseudoxanthoma elasticum, i.e. a homozygous *Abcc6* null mutant (Klement et al., 2005), but the same does not seem to hold true for elastic fibers in the human disease, whereas early calcification of microfibrils has been described for dystrophic cutaneous calcification (Fartasch et al., 1990). The molecular basis of elastic fiber calcification in these disparate conditions and the possible role of fibrillin are at present unclear.

Several findings suggest that dominance in Marfan's syndrome is caused by adverse activity of the mutant protein on the deposition, stability, or function of the protein encoded by the normal copy of *FBN1* (dominant-negative effect). Alternatively, half-normal production of the normal protein (haploinsufficiency) might be critical to reach the threshold loss of fibrillin-1 function needed for clinical expression of the disorder. Although the reduction in microfibrillar abundance due to haploinsufficiency may or may not be sufficient to lead to classic Marfan's syndrome, it determines a context within which a dominant negative effect can achieve clinical significance. The distribution analysis of the mutations shows a lower frequency of mutations in exons 45 and 57, while a higher number of mutations have been identified in exons 13, 26, 27, 28 and 43 (Tiecke et al., 2001). The most frequently observed mutations are missense mutations affecting cbEGF motifs, which can be further classified according to their expected effects on fibillin structure and function. Mutations of the highly conserved cysteine residues of the cbEGF-like motifs, or mutations introducing "extra" cysteins are likely to cause domain misfolding, which may have serious effects on the structure of fibrillin. Mutations affecting

residues of the calcium binding consensus sequence may result in reduced affinity for calcium and cause destabilization of the interface between two cbEGF-like domains (Downing et al., 1996). Mutations in cbEGF motifs not affecting cysteines or residues of the calcium binding consensus sequences are rare, and have been postulated to affect intra- or intermolecular interactions. In addition to these, missense mutations (premature truncation codon mutations and mutations associated with exon skipping) have also been identified in other motifs (Collod-Béroud et al., 1998). Calcium binding is essential for microfibrillar organization and integrity. Calcium binding is predicted to introduce a local conformational change in the N-terminal portion of cbEGF modules; mutations in these binding sites lead to conformational changes due to low calcium affinity and also to the new aminoacid itself (Downing et al., 1996). Calcium protects wild-type fibrillin-1 from proteolysis; similar findings have been obtained for a range of other matrix molecules. As shown in vitro, *FBN1* mutations can reduce the calcium affinity of cbEGF motifs and can increase the susceptibility of fibrillin peptide to proteolysis by exposing enzyme-specific cryptic cleavage sites (Booms et al., 2000; Reinhard et al., 1997; 2000).

CLINICAL MANIFESTATIONS OF MARFAN SYNDROME

Marfan syndrome involves primarily the cardiovascular, skeletal and ocular apparatuses, the meninges (with dural ectasia) and skin (with relatively small and benign manifestation); the respiratory apparatus usually shows a minor involvement, but some patients may develop pneumothorax and apical bullous emphysema (fig. 3) (Wood et al., 1984; Robbesom et al., 2008). The high morbidity of patients with MFS is mainly due to cardiovascular pathology (aortic dissection and dilatation, mitral valve prolapse, mitral and aortic valve regurgitation); aortic dissection alone is responsible for almost 80% of deaths (Murdoch et al., 1972, Silverman et al., 1995). Atrophic striae are the most common skin manifestation, affecting about 65% of subjects (Pyeritz, 2002). These striae are usually located in the body districts subjected to tension, such as buttocks, breast, thighs and abdomen (Cohen and Schneiderman, 1989), and are not associated with weight changes, pregnancy or excessive stress (Glesby and Pyeritz, 1989). Skin is often thinner than normal and the underlying fat tissue is reduced (Pyeritz, 2002). Knowledge about the behavior of skin in MFS are relatively scarce, despite the role that microfibrils play in this tissue.

Marfan-related structural abnormalities in the cardiovascular apparatus

The correlations between the type of genetic defect and the ultrastructural phenotype, in particular of blood vessels, are at present unknown. Regardless of genotype, with the passage of time the wall of the ascending aorta is subjected to constant stress which in patients with MFS determines dilatation. The attempt to repair the tear by smooth muscle cells is insufficient and eventually the wall rup-

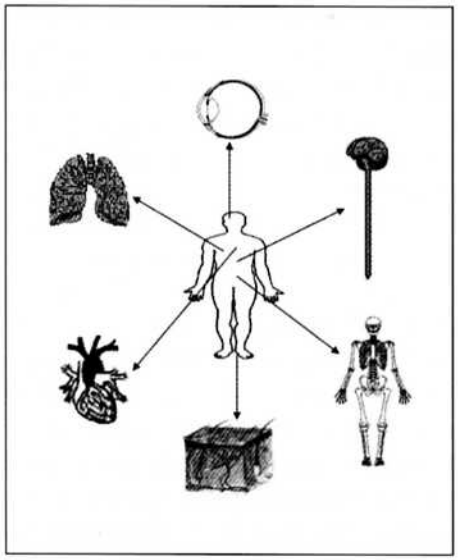

Fig. 3 — Organs and systems involved in Marfan syndrome. Clockwise, from above: eye, central nervous system and specifically meninges, skeleton, skin, heart and vessels, lungs.

tures. In one of the first studies on microfibril ultrastructure, Scheck et al. (1979) studied the aortic aneurysm wall and mitral valve tissue a patient with MFS and found elastic fiber degeneration, increase in number and morphologic alterations of collagen fibers, and the presence of smooth muscle cells with synthetic phenotype. In particular, the surface irregularities of elastic fibers, connecting them with myocells by means of a microfibrils layer, were extremely few and had the form of plugs instead of spikes; in advanced stages elastic fibers were disrupted. The collagen fibrils showed marked heterogeneity in size, irregular profiles (embossed or indented), and aggregation into broad, complex aggregates with typical banding that have a flower-like appearance in cross-section. These results underscore the importance of the extracellular matrix, in general, and of elastic fibers, in particular, for the strength of aortic tissue. Bunton et al. (2001) have analyzed the aorta of mice homozygous for a mutation in *FBN1* and have found fragmentation and calcification of elastic fibers and lack of connection between microfibrils and smooth muscle cells. The authors propose that Fbn1 deficiency leads to loss of cell attachment mediated by microfibrils and that this would be a signal to stimulate the turnover of the extracellular matrix with eventual prevalence of the synthesis of un-elastic structures and partial resorption of elastic sheets.

Atrioventricular valves are frequently altered in extracellular matrix composition and organization in MFS, particularly regarding collagen and proteoglycans. Ng et al. (2004) implied a causal relationship between aberrant activation of TGF-β by non-functional Fbn1 and the valve myxomatosis typical of MFS: the cytokine

may promote the secretion of an altered extracellular matrix which would be responsible for the mixoid degeneration. In a mouse model with defects in *Fbn1*, Neptune et al. (2003) observed TGF-β hyper-activity, which was considered the basis for the tendency to develop emphysema and perhaps for the manifestations of MFS. Disruption of TGF-β signal can then contribute to the pathogenesis of extracellular matrix disorders. Carta et al. (2006) have obtained data which suggest that a continuous Fbn1 deposition is necessary for correct development of mouse aorta during neonatal life. Ramirez and Dietz (2007) have studied the role of fibrillin during prenatal development using mutant mice and have suggested that at this stage fibrillin binds growth factors (TGF-β and BMP) in certain positions, perhaps ensuring concomitant adhesion-mediated signaling and enhancing growth factor signaling. Organisms with only mild deficiency in fibrillin would not have defects in development but would suffer from the effects of excess negative signaling, perhaps even ill-localized, during postnatal life; in this latter period of life, damage would arise from failure to link growth factors and consequently to damp their signal. On the basis of their findings and of literature data, Ramirez and Dietz (2007) have drawn attention to "TGF-β signaling" in development and in some neonatal genetic arteriopathy (regardless of changes in fibrillin) and have envisaged a possible use of TGF-β antagonists in these syndromes.

Niwa et al. (2001) have studied the wall of large vessels in various malformations, finding fragmentation and loss of elastic fibers and increase in collagen of varying severity. It is not possible to decide how many of these changes are related to the cause of the defect (i.e., altered cellular function that determines both altered microscopic organization and distorted anatomical architecture) and how many are secondary to incongruous mechanical load imposed to the wall by anatomical malformations. Similar doubts may be prospected for cases of MFS. Dingemans et al. (2006) have analyzed biopsies taken at surgery for aorta rupture from patients with MFS and from control patients, also operated for aorta rupture but without the syndrome. These studies have revealed the presence of interrupted elastic plates, prevailing near the dissection areas and limited to older patients, and a decrease in the connections between elastic sheets and smooth muscle cells; these connections – as mentioned above – are formed by protrusions of the elastic fibers and by microfibrils. The question why there is an uneven distribution of the microscopic disruptions of elastic fibers in aortic biopsies remains open; these alterations are more abundant in areas of greatest tension and this may be one causal element for dissection in these areas. Another point to clarify is the mechanism that, in patients with MFS, leads to a decrease with age in the number of connection spikes between elastic fibers and smooth muscle cells; one hypothesis is that with time the cell capacity of synthesis is overcome, and cannot cope with an accelerated regeneration of the extracellular matrix required by the defect. Fibrillin deficiency may also lead to early decline in number of the cells that synthesize this protein: Nataatmadja et al. (2003), using histophotometric measurement techniques in cell culture in parallel with Western Blot analysis to assess the production of extracellular matrix molecules, have provided re-

sults in favor of the hypothesis that fibrillin protects fibroblasts and vascular smooth muscle cells from apoptosis, since vascular smooth muscle cells from patients with MFS show in culture an abnormal high rate of apoptosis.

Other components, in addition to the elastic component, are important for vascular wall stability. Heegaard et al. (2007) found abnormalities of the aortic extracellular matrix in a model of mutant mice lacking the gene for biglycan, so that in half of the males there was spontaneous rupture of this vessel. There were also changes in collagen fibrils, consisting in extreme heterogeneity in diameter and irregularity of the profile with corrugated or indented contour. Biglycan is also present in elastic fibers, without a specific location in the various portions of these fibers (Baccarani-Contri et al., 1990), so it is possible that changes described in the mutant mice without biglycan may depend at least by an interference with the structural integrity or at least with the functional efficiency of elastic fibers.

Marfan-related structural abnormalities in the skin

The correlations between the genetic defect of *FBN1* gene and the ultrastructural phenotype of skin are not yet known. Pieraggi-Fabre and Bouissou (1971) have studied the dermis of 4 patients with MFS and of 1 patient with homocystinuria. The ground substance was extremely abundant in the superficial dermis where cells were few and sparse and elastic fibers were also few and looked worm-eaten and frayed and with dense matrix. In the deep dermis there were only a few, abnormal elastic fibers: they were fragmented, jagged, inhomogeneous in thickness and staining. Collagen bundles in superficial dermis were small, short and often dissociated into individual thin fibers; normal collagen bundles in the deep dermis looked normal. There was also a pseudo-elastic material of dubious origin. Fibroblasts showed degenerative changes represented by cytoplasmic matrix darkening, mitochondrial condensation alternated to dilatation, presence of vacuoles and electron dense inclusions interpreted as lysosomes; the cells which did not show these changes were surrounded by flocculent material that, at high resolution, appeared grainy and not organized into fibrils. The authors drew attention to the alterations of fibroblasts and suggested two possible explanations: (a) the lack of fiber polymerization may lead to a relative increase of the ground substance and in turn to a resentment of fibroblasts responsible for a quantitatively increased but qualitatively defective secretory activity; (b) the observed changes may be expressive of primary fibroblast sufference, leading to abnormal secretion of extracellular matrix molecules unable to polymerize correctly. Kobayasi (2006) analyzed skin biopsies from female patients with hypermobile disorders, aged between 1 and 80 years. They reported progressive structural deterioration with age: disorder in microfibril arrangement, followed by their disappearance in old people; occurrence of stripes that interrupt the homogeneity of elastic fiber matrix (which normally is thin and transparent in childhood and progressively more abundant and dense with age); thickening of the amorphous surface coating of elastic fibers (which is made of glycosaminoglycans);

irregular outline of these fibers, worsening with advancing age; and fiber disintegration in old people. Women with MFS exhibited abnormalities similar to those seen with aging but much earlier, so that microfibrils were already absent in adulthood, and at this same age the elastic fibers appeared degenerated, with moth-eaten aspect or with several indentations on the edge.

CONCLUSIONS

Fibrillin, besides playing a structural role in elastic and oxytalan fibers, interacts with several growth factors and morphogenetic proteins, stabilizes matrix components and keeps contacts between the extracellular matrix and cells. Assembly of fibrillin monomers into microfibrils and of microfibrils into elastic fibers are complex processes, not yet fully understood. Fibrillin behavior depends on factors related to fibrillin itself and to the environment in which it is inserted; a special role is played by calcium, which binds to specific domains of fibrillin and regulates their conformation and hence the assembly and functional performance of fibrillin. Mutant fibrillin monomers may influence protein folding, stability and proteolysis of both the mutant and normal protein, microfibrillar assembly and fragmentation, anchorage between extracellular matrix and cells with the intermediate of basal lamina, and TGF-β signaling. The loss of cell-matrix interactions, as well as the disruption of microfibrils, are likely to underlie the pleiotropic manifestations of MFS. This syndrome may therefore provide a model to understand the role of fibrillin, and more generally of microfibrils, in the extracellular matrix organization, and the correlation of this matrix with the cells immersed in it and responsible for its synthesis. A complete understanding of the pathogenesis of MFS requires further advances in many different areas and will presumably take advantage of recognizing how different mutations cause different disease phenotypes. In turn, achievements in those areas will increase our knowledge of roles of the microfibrils and of extracellular matrix in general in organ formation and function, as well as improve the clinical management of connective tissue disorders.

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